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FOREWORD

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I. Introduction

The *c-kit* gene is the cellular homologue of the transforming gene of a feline sarcoma virus (1) encoding a transmembrane tyrosine kinase growth factor receptor in the same sub-class as the receptors for platelet-derived growth factor and colony stimulating factor-1 (2). Its ligand, alternatively known as stem cell factor (SCF), mast cell growth factor, *kit* ligand, or *steel* factor, is a hematopoietic growth factor that, in conjunction with other hematopoietic growth factors, supports the proliferation and differentiation of multiple hematopoietic cell lineages(3-5). In vitro, SCF has only a modest effect on proliferation of early hematopoietic precursors, exerting its primary influence as it potentiates the effects of other growth factors, including interleukin 3 (IL-3), erythropoietin, granulocyte-macrophage colony-stimulating factor (GMCSF), and granulocyte colony-stimulating factor (GCSF) (6-9).

c-kit and SCF coexpression has been described in a number of malignancies, including breast cancer. *c-kit* is over expressed in testicular germ cell tumors (10), melanomas (11), leukemic blasts (12-14) and also in a glioblastoma cell line (2). Leukemic blasts and the glioblastoma line can be growth stimulated by exogenous SCF (15) while SCF is inhibitory for some melanoma cell lines (11). *c-kit* and SCF are coexpressed in the majority of small cell lung cancer cell lines (16-18), as well as in the human neuroblastoma (19) suggesting the presence of an autocrine loop.

For breast cancer, *c-kit* and SCF coexpression was first noted in the Du4475 cell line (20) We have since shown the Kit receptor and its ligand are, in fact, coexpressed in a majority of surgical breast tumor specimens as well as in the majority of established breast cancer cell lines(21).

We have proceeded to investigate some of the biologic consequences of *c-kit* and SCF coexpression since simple coexpression of receptor and ligand may imply, but does not prove, a potential autocrine(or paracrine) loop. We have therefore, constructed an autocrine loop by transfecting the breast cancer cell line, MCF7, that only expresses SCF, with a *c-kit* expression vector (21). MCF7 cells were transfected with both the *c-kit* expression vector and the empty pCEP4 vector (to serve as a control). Since the vector contained a hygromycin resistance gene, stable transfectants could be selected based on their survival in hygromycin-containing media.

From the original pooled *c-kit* transfectants, three subclones expressing variable levels of Kit were selected (Figure 1). KIBI had the highest level of Kit expression, KVAI the lowest, and KIIA4 intermediate. They were matched with control subclones CIC2 (KIBI), CIC3 (KIIA4), and CIA2 (KVAI) based on similar number of copies of hygromycin resistance genes (as determined by Southern blotting). Despite varying levels of total Kit expression, the degree of tyrosine phosphorylation was similar among the subclones, suggesting the presence of either limiting amounts of endogenous SCF or regulation of the amount of Kit phosphorylation by a cellular phosphatase.

In serum-free medium containing defined growth factors, the growth of *c-kit* transfectants exceeded that of their controls. This growth advantage was seen in the presence of EGF, IGF1, (Figure 2A, 3A) and heregulin (not shown). Interestingly, addition of exogenous SCF (100 ng/ml) induced growth only in the highest *c-kit* expressing subclone, KIBI. It is possible that KIBI's isolated response to SCF may reflect that , in contrast to the subclones expressing lesser amounts of Kit where the receptors are perhaps saturated by endogenous SCF, only in a high level Kit expresser is there an excess of a receptor to respond to exogenous SCF. This effect was additive in the presence of IGF1 (Figure3A). In contrast, KIBI's response to SCF was not further increased in the presence of EGF(Figure 2B). These responses were certainly Kit mediated as the SCF- induced growth was partially inhibited by a monoclonal blocking antibody directed against Kit (Boehringer-Mannheim, Indianapolis, IN) (Figure 3B).

We have shown that the breast tumor cell line ZR-75-1 represents a naturally occurring example of *c-kit* and SCF coexpression. Similar to the response seen in the Kit-expressing subclones, ZR-75-1 shows growth in serum-free medium containing IGF-1 or EGF. Addition of the Kit blocking antibody inhibits IGF-1 induced growth by \approx 50%. Similarly, EGF stimulated growth is reduced nearly to the level seen in serum-free medium alone.

In order to understand *c-kit* 's contribution to growth in both normal and in malignant systems we must have knowledge of its signal transduction pathway .The major pathway utilized by the tyrosine kinase growth factor receptors employs the Ras protein to transmit signals from the membrane-bound receptor through the cytoplasm to the cell nucleus (22-24). The basic scheme begins with ligand binding to the tyrosine kinase receptor inducing both transphosphorylation and autophosphorylation on tyrosine residues. Several of the cytosolic proteins subsequently activated (including SHC, GRB-2, Ras-GTPase-activating protein) contain regions of homology to a sequence of 100 amino acids in the noncatalytic domain of pp60^{c-sr} - known as the SH2 domain (25). Possession of SH2 domains allows direct binding of these signal transduction molecules to receptors activated through tyrosine phosphorylation.

Following ligand stimulation of the tyrosine kinase receptor, GRB-2 binds directly (or indirectly via its interaction with SHC) to the phosphorylated receptor and recruits the SOS protein (a guanine nucleotide releasing factor) to interact with Ras at the plasma membrane. Prior to interacting with SOS, Ras exists inactive, bound to GDP. Coupling to SOS exchanges GDP for GTP and Ras is activated. Downstream, the Raf protein forms a bimolecular complex with activated Ras, first leading to activation of mitogen-activated protein kinase kinase (MAPKK) and next to activation of mitogen-activated protein kinase (MAPK). This cascade culminates in phosphorylation of transcription factors within the cell nucleus.

The Ras-MAPK pathway is currently the best characterized signal transduction pathway. A second pathway, utilized by the platelet-derived growth factor receptor, involves ligand-induced autophosphorylation of the tyrosine kinase receptor and recruitment of phosphatidylinositol 3-kinase (PI3-kinase) (22,23). Phosphatidylinositol-specific phospholipase C-γ (PLC γ) also becomes associated with the receptor, likely via SH2 domains. This in turn induces generation of diacylglycerol and inositol-1,4,5-triphosphate resulting in activation of protein kinase C and release of intracellular calcium (22).

The above schemes have resulted primarily from study of the platelet-derived growth factor receptor and the epidermal growth factor receptor. Kit's mechanisms of signal transduction are beginning to be worked out - to date, most work has been done in hematopoietic systems. SHC, with its single SH2 domain, has been shown to undergo tyrosine phosphorylation in response to SCF (26,27) and additionally, associates with GRB-2 (27) under these conditions. For GAP, association with Kit is not consistently observed (28-30) nor is interaction between Kit and PLC γ (28). Both Raf-1 and MAPK are phosphorylated in response to SCF stimulation (28,31-33). Protein kinase C phosphorylates the activated Kit receptor, inhibiting SCF-induced autophosphorylation on tyrosine, thus creating a negative feedback loop - decreasing kinase activity without changing ligand binding (34). Not only is signaling mediated through the stimulatory effects of tyrosine phosphorylation but also through the inhibitory actions of tyrosine phosphatases. Hematopoietic cell phosphatase (HCP or PTP1C or SHPTP1) has been shown to transiently associate with the stimulated Kit receptor, via its SH2 domains, in leukemic cells (35).

While Kit does seem to play a role in signal transduction in hematopoietic systems, no work is yet published outlining Kit's role in the signal transduction pathway of benign or malignant breast tissue. Understanding the signaling pathways through which the processes of proliferation and differentiation are mediated may enable development of inhibitors that could be valuable in therapeutic strategies (36).

II. Methods

A. Cell Growth. For growth studies, ZR-75-1 cells and MCF7 cells transfected with the c-kit expression vector (21) were grown in RPMI 1640 supplemented with 2mM L-glutamine, 10⁻⁹M estradiol and 0.1% bovine serum albumin (Sigma, St. Louis, MO). Otherwise, cells were maintained in RPMI 1640 containing 10% fetal calf serum(Life Technologies, Inc., Gaithersburg, MD) and, for the Kit-transfected cells, hygromycin B 200 µg/ml (Calbiochem, San Diego, CA). Where indicated, serum-free medium was supplemented with recombinant IGF-1 (R&D Systems, Minneapolis, MN), EGF (R&D Systems, Minneapolis, MN), SCF (Biosource, Camarillo, CA). Cell growth was measured using the 3-(4, 5 dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (Sigma, St. Louis, MO) dye reduction method (37). Preliminary experiments indicated that dye reduction was proportional to viable MCF7 cell number under the conditions used. Eight replicate wells per time point were plated at a density of 5 x 10³ cells and

data was expressed as the increase in absorbance at 540 nM over time, relative to initial values obtained three hours after plating. Tyrphostin AG1296 (Calbiochem, San Diego, CA) was dissolved in DMSO with a final DMSO concentration of 0.1% in all conditions, including controls.

B. Western Blotting and Immunoprecipitation.

Cells were pre-incubated overnight in serum free medium, then stimulated with the appropriate growth factor for the time indicated. Western Blotting was performed according to Sambrook, *et al* (38) using 50-250 µg of total cellular protein. Transfer to nitrocellulose membranes was monitored using prestained molecular weight markers. Staining was accomplished using the following antibodies: antiphosphotyrosine - horseradish peroxidase conjugate, RC20H (Transduction Labs, Lexington, KY); Stat 5 (UBI, Lake Placid, NY); rabbit polyclonal Stat 5b, rabbit polyclonal Jnk1 and Jnk2, rabbit polyclonal IGF1R β (Santa Cruz, Santa Cruz, CA); mouse monoclonal EGF R Ab, rabbit polyclonal anti rat MAP kinase R2, (UBI, Lake Placid NY); c-erB4/Her4 oncprotein Ab2 (Neomarkers-LabVision, Fremont, CA); polyclonal rabbit anti active MAP kinase (Promega, Madison, WI). Visualization was accomplished using the ECL detection system (Amersham, Arlington Heights, IL).

Immunoprecipitation was accomplished by lysing cells in RIPA buffer [150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50mM Tris (pH 8.0)] containing 100 µg/ml phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, and 0.2mM sodium vanadate, incubating the lysate with the appropriate IP antibody for 2 hours at 4°C, and then adding protein A+G agarose for an additional 2 hour incubation. Antiphosphotyrosine immunoprecipitation used the antiphosphotyrosine -agarose conjugate (PY20) (Transduction Labs, Lexington, KY) and IGF1R immunoprecipitation required the mouse monoclonal anti IGF1R (Oncogene Science, Uniondale, NY).

III. Results

A) "Determine the active members of Kit's signal transduction pathway in breast cancer."

As described above, the KIBI transfecant with the highest expression of Kit protein, responds to exogenous SCF with increased growth. This subclone also responds to both IGF1 and EGF administration. However, the responses to these growth factors, when combined with SCF, show different characteristics. Growth in the presence of SCF + IGF1 is additive - together, growth is greater than with either growth factor alone. In contrast, while KIBI shows growth in response to SCF and to EGF, SCF + EGF growth is no greater than that with either growth factor alone. One hypothesis that could explain these observations is that Kit and IGF1 could mediate their growth signals via different pathways and thus achieve additive growth. If, however, Kit and EGF were to utilize the same signaling pathway, the pathway could become saturated and thus there would be an upper limit on the amount of growth that could be induced.

1) Does the amount of tyrosine phosphorylation of the EGF or IGF1 receptor change in response to SCF stimulation?

We first looked at KIBI cells with and without SCF stimulation (100 ng/ml). MCF7 cells have been previously described as expressing very small amounts of the EGF receptor (39). Immunoprecipitation with the anti-phosphotyrosine antibody and western blotting for the EGFR failed to show any quantitative difference in tyrosine phosphorylation between SCF stimulated and non-stimulated cells. The converse experiment was also performed - immunoprecipitating the EGF receptor and western blotting with anti phosphotyrosine. This study preliminarily suggested no difference in phosphorylation, but awaits repeat analysis. Not only did tyrosine phosphorylation appear equal, but there was no demonstrable quantitative difference in amount of EGF receptor as determined by immunoprecipitating with an anti-EGFR antibody and then western blotting with same antibody. Antiphosphotyrosine immunoprecipitations performed and stained with anti-erbB2 and anti-erbB4 antibodies also failed to detect any quantitative differences in tyrosine phosphorylation between SCF stimulated and non-stimulated cells.

To look at the IGF1 receptor, again, the KIBI cells were lysed in the presence or absence of SCF. Western blotting was performed on total cell lysate using an antibody directed against IGF1R β . No difference in level of IGF1 receptor was observed in comparing SCF stimulated and non-stimulated cells.

Immunoprecipitation with anti-IGF1R and western blotting with anti-IGFR β showed no difference in amount of receptor. Finally, immunoprecipitation with the anti-phosphotyrosine antibody and western blotting with anti-IGF1R β showed no difference in tyrosine phosphorylation of the IGF1 receptor in response to SCF stimulation.

2) Are there differences in patterns of tyrosine phosphorylation among KIBI cells stimulated with EGF versus IGF1 versus SCF?

To begin to answer this question we looked at KIBI cells in the presence of EGF (100 ng/ml) or IGF1 (100 ng/ml) or SCF (100 ng/ml). Cells were analyzed following 0, 1, 5, 10 and 15 minutes of growth factor stimulation. Western blotting of total cell lysates used the antiphosphotyrosine antibody (RC20H).

a) Comparison of tyrosine phosphorylation among the three conditions showed strongly phosphorylated bands in the 40-50 kD range. Members of the MAP kinase family fall in this size range, including a member of the MAP kinase family, designated c-Jun NH₂-terminal kinase (JNK) with a molecular weight of

54 kD. Probing the total cell lysate with anti JNK1 and anti JNK2 antibodies showed a constant level of tyrosine phosphorylated JNK1,2 protein throughout the 15 minute time course and there were no differences observed among the SCF, EGF, or IGF1 stimulated lysates.

Next, we probed the total cell lysates with an antibody directed against total MAP kinase. Not only were we looking for the expected 42 and 44 kD bands, but also hoped to detect protein migrating at a slighter higher molecular weight, consistent with tyrosine phosphorylation of the MAPK protein. The two MAP kinase forms were present in equivalent amounts throughout the time course and did not differ based on EGF, IGF1 or SCF stimulation. No shift in protein mobility was detected to suggest phosphorylation.

Our original assay used cellular lysates in which the nuclear material had been discarded. Since the MAPK signaling proteins are responsible for bringing the signal cascade to the nucleus, perhaps we had eliminated the major source of MAPK proteins. Therefore, we repeated the assays using whole cell protein lysates. Cells were lysed after a fifteen minute incubation with either EGF, IGF1, SCF OR EGF + SCF, IGF1+ SCF. Similar experiments were performed using the KIBI, high Kit protein expresser, and KVAI, the lowest Kit expresser.

Now, Western blotting of the whole cell lysates with anti active MAPK does show some differences among the various stimuli and also between the KIBI and KVAI cell lines. Not surprisingly, KIBI + SCF appears to activate MAP kinase following a fifteen minute incubation (Figure 4A). In contrast, KVAI + SCF has a low relative level of active MAPK (Figure 4B), comparable to that in unstimulated KVAI cells. This would be in keeping with what we saw in the growth assays. Only KIBI responds to exogenous SCF with increased cell growth. In both the KIBI and KVAI lysates, MAPK is activated in the presence of IGF1 and EGF. It must be noted that these MAPK assays utilizing whole cell lysates, are preliminary. These blots must yet be reprobed with total MAPK to assure that protein loading is equivalent. Also, we plan to probe the whole cell lysates for the presence of any quantitative differences in the IGF1 and EGF receptor. Finally, we will investigate a time course for MAPK activation and see if differences exist related to the growth factor stimulus involved.

b) In comparing total cell lysates of KIBI cells stimulated with IGF1 or SCF and then stained for antiphosphotyrosine, there is a phosphorylated protein in the 80-90kD range that appears in the KIBI cells stimulated with SCF, at 10 and 15 minutes (Figure 5). It does not appear in the IGF1 stimulated lysate. Stat 5 (signal transducer and activator of transcription) has a MW of approximately 90 kD and is part of the JAK-STAT signaling pathway originally described as the mechanism of signaling for interferon α . Interestingly, Stat 5 was originally

identified as a mammary gland factor responsible for binding to promoter sequences of milk protein genes and activating their transcription (41,42). Probing the cell lysates with an anti Stat 5 antibody demonstrated similar amounts of Stat 5 protein at all time points and there was no significant difference between the IGF1 and SCF stimulated cells. Next we performed an immunoprecipitation with antibody directed against Stat 5b and western blotted for antiphosphotyrosine. There was no tyrosine phosphorylation of Stat 5b following 15 minutes of EGF, IGF1, or SCF stimulation (Figure 6A). Stripping and reprobing with anti STAT 5b confirmed equal amounts of protein in all lanes (Figure 6B).

So, if Stat 5b is not responsible for the 90ish kD band, peaking in the SCF stimulated cells, what other signaling proteins might migrate at this weight? PI3 kinase with a molecular weight of 85 kD, is next being assessed.

B) "Determine the growth factors that cooperate with Kit/SCF interactions."

Tyrphostin AG1295 is a protein kinase inhibitor known to be selective for the PDGF and Kit receptors. Since MCF7 cells are known NOT to express the PDGF receptor (42), inhibition of cell growth in the presence of tyrphostin AG1295 must be mediated through the Kit receptor. Previous growth studies utilizing MCF7-kit transfectants in the presence of tyrphostin AG 1295 revealed that at 10 μ M tyrphostin, growth of the KIBI transfectant could be reduced to that of the control cells, indicating complete inhibition of intra- and extracellular Kit/SCF interactions. With the availability of tyrphostin AG1296, thought to be even more specific for Kit than was AG1295, we repeated some of the growth analyses. In testing other breast cancer cell lines for sensitivity to tyrphostin AG1296, we did not find any consistent results among cell lines such including BT20, T47D, MCF7, SKBR3. The highest concentration of tyrphostin AG1296, that still remained soluble, was 20 μ M. Both KIBI cells and the matched control line, CIC2 were inhibited in the presence of tyrphostin AG1296. This could not be overcome with SCF administration. However, the fact that growth of both control and Kit transfectants was inhibited by tyrphostin AG1296 suggests that, in these cells, tyrphostin AG1296 is not specifically acting through the Kit receptor.

IV. Discussion

We have previously shown c-kit and SCF to constitute a unique autocrine loop (21). MCF7 cells , that naturally express SCF, can be transfected with a c-kit expression vector, and these cells demonstrate enhanced growth in serum-free medium. The growth of these cells can be further augmented through supplementation with individual growth factors, including EGF, IGF1(21) and heregulin (in preparation). The fact that this growth is related to Kit expression is illustrated by the ability of an anti-Kit blocking antibody to eliminate growth in the presence of EGF and IGF1. These changes in

growth are not unique to our artificial system but are also seen in the ZR751 cell line that naturally coexpresses Kit and SCF (21). Interestingly, for the MCF7-KIBI subclone that expresses the highest level of Kit protein, and demonstrates responsiveness to exogenous SCF, the growth pattern in the presence of SCF + IGF1 is additive. This contrasts with the growth produced through EGF + SCF which does not exceed that seen with EGF stimulation alone. We hypothesize that this may reflect differences in patterns of signal transduction. If, for example, Kit and IGF1 produce their effects on growth using distinctly separate pathways, growth additivity could be an expected result. However, if Kit and EGF mediate growth through the same signaling cascade, the pathway could become saturated, thus placing an upper limit on growth. Ultimately, being able to explain these differences could provide insights that might have therapeutic influences. The farnesyl transferase inhibitors have already shown that exploitation of cell signaling mechanisms can result in therapeutic advances (43).

To begin the investigation of Kit's signaling pathway in breast cancer, we used the SCF-responsive KIBI subclone to see whether there were any changes in quantity or quality of EGF and IGF1 receptors in response to SCF administration. Neither quantity of EGF or IGF1 receptor nor amount of tyrosine phosphorylation was altered in response to SCF.

We next compared KIBI cell lysates stimulated with EGF or SCF or IGF1 and looked for any differences in patterns of tyrosine phosphorylation. Prominent phosphorylation was present in the 40-50kD range and this suggested MAP kinase activity. Previous work in hematopoietic systems has shown Raf-1 and MAPK phosphorylation in response to SCF (28,31-33,44). Although cytoplasmic lysates failed to show significant differences in MAPK levels, whole cell lysates that retained nuclear material were more informative. Here we saw MAPK activity to reflect what our previous growth studies had revealed. Only the KIBI subclone that expressed very high levels of Kit protein showed increased growth in response to SCF. This was reflected in increased levels of active MAPK. In contrast, the KVAI subclone that expressed the lowest level of Kit receptor and failed to respond to SCF, showed a level of active MAPK that was comparable to that seen in the unstimulated KVAI cell line.

In comparing KIBI cell lysates stimulated with IGF1 versus SCF, there was a prominent and reproducible difference in tyrosine phosphorylation in the 90 kD range. In SCF stimulated lysates a band appeared following 10-15 minutes of stimulation and this band was absent in IGF1-treated KIBI lysates. Our first thought was that this might represent Stat5 (MW 95) but immunoprecipitation of the Stat5 protein failed to show detectable tyrosine phosphorylation. Conflicting literature exists regarding activation of Stat in response to SCF stimulation. Kit has been shown to associate with the erythropoietin receptor (45), a receptor known to activate JAK2 thus engaging the JAK-STAT pathway (46). Recently Ryan, et al (47) utilized electrophoretic mobility shift assays to show that SCF stimulation of mouse bone marrow-derived mast cells

led to a rapid and transient activation of a DNA-binding factor ultimately identified as Stat5. However, prior to this report, publications of Pallard and O'Farrell (48,49) failed to show Stat5 activation in response to SCF. Pallard et al used a megakaryocytic cell line stimulated with 50 ng/mlSCF for 20 minutes and observed no Stat5 activity. Ryan, et al suggests that this longer period of SCF stimulation may in fact be too long to detect Stat5. Although our Stat5 immunoprecipitation used cells treated with 15 minutes of EGF or IGF1 or SCF, we had stained cell lysates for Stat5 protein over a 15 minute time course and found no detectable differences in amount of protein. This does not, however, address the time course of phosphorylated Stat 5 and so, performing a Stat5 immunoprecipitation of cells treated with 1, 5, 10 and 15 minutes of growth factor and then staining the IP's with anti-phosphotyrosine, may answer this question. However, it would not explain the phosphorylated 90ish kD band that does not appear until 10-15 minutes in the KIBI cells treated with SCF. And, therefore, it is still reasonable to assay for PI3 kinase (MW 85). As Ryan, et al, also points out, his evidence of SCF induced Stat 5 activation occurs in seven independent IL-3 cultured bone marrow derived mast cell lines and the two negative studies, as well as our own, focus on Stat5 activity within a single cell line. Depending on the results of the time course of Stat5 tyrosine phosphorylation it may be reasonable to similarly assay the ZR-75-1 cell line for Stat5 activation.

V. Conclusion

We have evidence that Kit and SCF constitute a unique autocrine growth loop. MCF7 cells that coexpress Kit and SCF also demonstrate enhanced growth in serum-free medium supplemented with individual growth factors including EGF and IGF1. Our current goals are to better characterize the signal transduction pathways that may be responsible for this growth and, in particular, for differences observed between IGF1 and EGF stimulated growth. In the MCF7 KIBI subclone that expresses high levels of Kit protein, there appears to be some differences in patterns of tyrosine phosphorylation between cell lysates stimulated with SCF vs IGF1. These differences focus on a 90ish kD phosphorylated band that does not yet seem to represent STAT5, although further investigation may be necessary. Prominent phosphorylation of signal proteins in the 40-50kD range may be explained by activation of the MAP kinase proteins. Preliminary data suggests that MAPK is activated in response to EGF and IGF1 ,and, in the transfected cell line KIBI where cell growth is induced by exogenous SCF, MAPK is activated by SCF.

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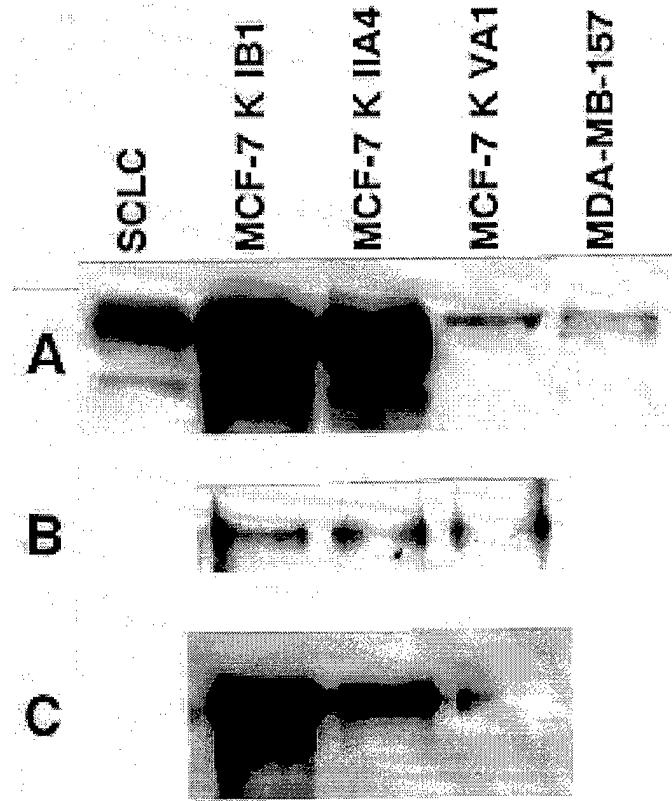
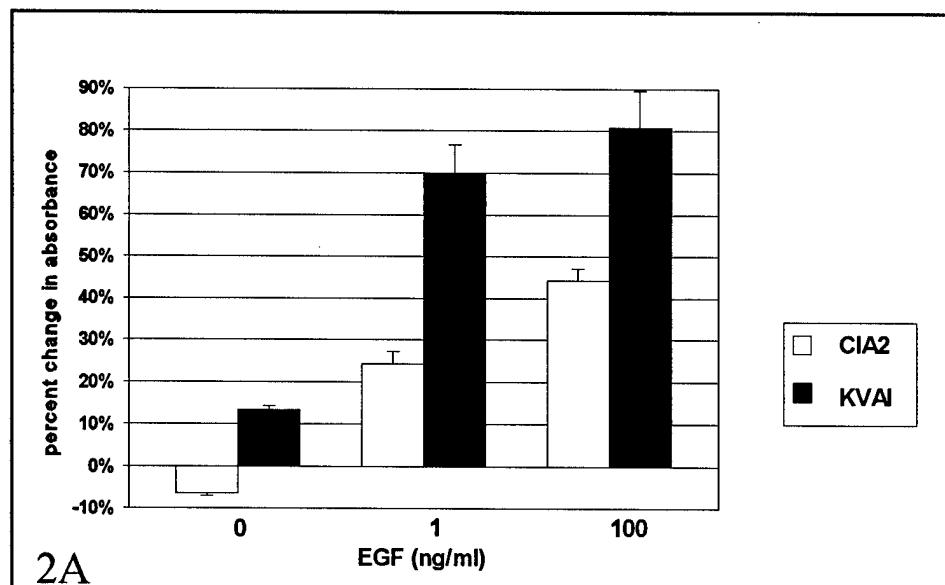
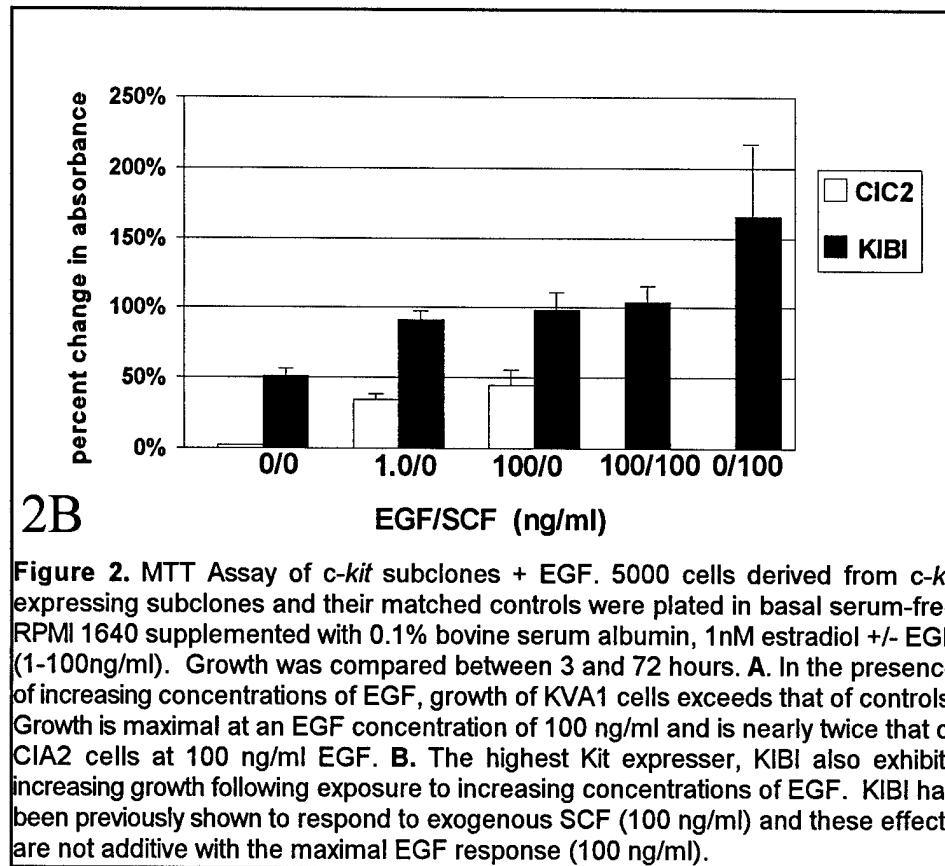


Figure 1. Western Blot analysis of MCF7 subclones expressing varying levels of Kit.
A. Western blot of 100 µg of total cell lysate from three *c-kit* transfected subclones, as well as the H510 line (small cell lung cancer) and MDA-MB-157 (40 µg), stained with a polyclonal Kit antibody. **B.** Western blot of Kit protein immunoprecipitated with a monoclonal Kit antibody and then stained with an anti-phosphotyrosine antibody. **C.** The same Western blot as in Fig. 1B. stripped and restained with the monoclonal Kit antibody, showing that recovery of the Kit protein is quantitative.



2A



2B

Figure 2. MTT Assay of c-kit subclones + EGF. 5000 cells derived from c-kit expressing subclones and their matched controls were plated in basal serum-free RPMI 1640 supplemented with 0.1% bovine serum albumin, 1nM estradiol +/- EGF (1-100ng/ml). Growth was compared between 3 and 72 hours. A. In the presence of increasing concentrations of EGF, growth of KVA1 cells exceeds that of controls. Growth is maximal at an EGF concentration of 100 ng/ml and is nearly twice that of CIA2 cells at 100 ng/ml EGF. B. The highest Kit expresser, KIBI also exhibits increasing growth following exposure to increasing concentrations of EGF. KIBI has been previously shown to respond to exogenous SCF (100 ng/ml) and these effects are not additive with the maximal EGF response (100 ng/ml).

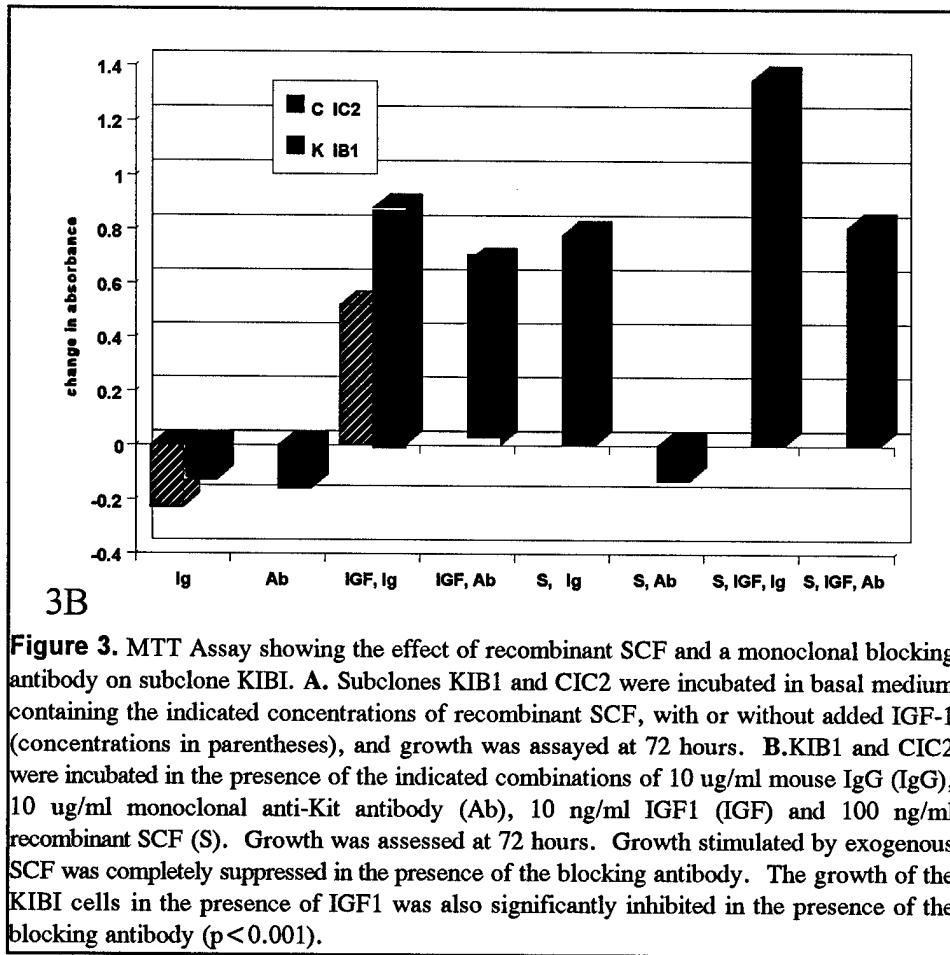
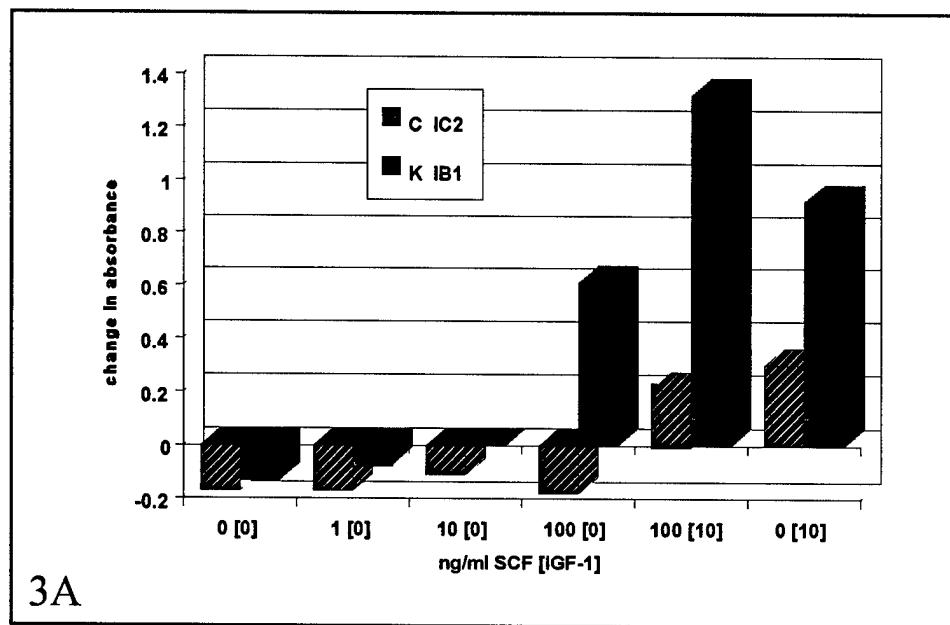


Figure 3. MTT Assay showing the effect of recombinant SCF and a monoclonal blocking antibody on subclone KIB1. A. Subclones KIB1 and CIC2 were incubated in basal medium containing the indicated concentrations of recombinant SCF, with or without added IGF-1 (concentrations in parentheses), and growth was assayed at 72 hours. B. KIB1 and CIC2 were incubated in the presence of the indicated combinations of 10 ug/ml mouse IgG (IgG), 10 ug/ml monoclonal anti-Kit antibody (Ab), 10 ng/ml IGF1 (IGF) and 100 ng/ml recombinant SCF (S). Growth was assessed at 72 hours. Growth stimulated by exogenous SCF was completely suppressed in the presence of the blocking antibody. The growth of the KIB1 cells in the presence of IGF1 was also significantly inhibited in the presence of the blocking antibody ($p < 0.001$).

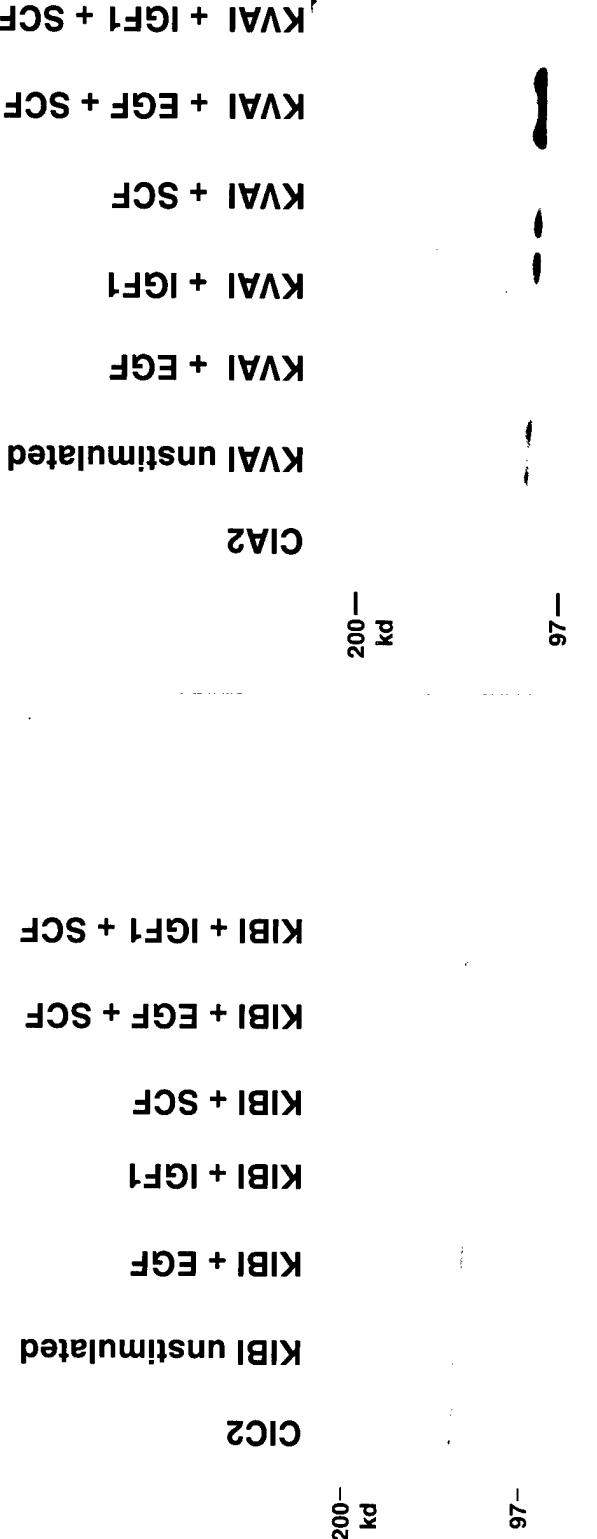


Figure 4. Western Blot analysis of KIBI and KVAl transfectants. Western blot of 250 µg of whole cell lysate from KIBI and KVAl subclones, following 15 minute stimulation with the appropriate growth factor and staining with a polyclonal rabbit anti-active MAP kinase antibody. The growth factors were (each at a concentration of 100 ng/ml): EGF, IGF1, SCF, or IGF1 + SCF. **A.** The KIBI whole cell lysates (subclone with highest level of Kit receptor) show MAP kinase is activated by SCF as well as IGF1 and EGF. **B.** In contrast to KIBI, the KVAl cells (lowest level of Kit receptor) do NOT appear to activate MAPK following SCF stimulation. Still, MAPK is activated in the presence of EGF and IGF1.

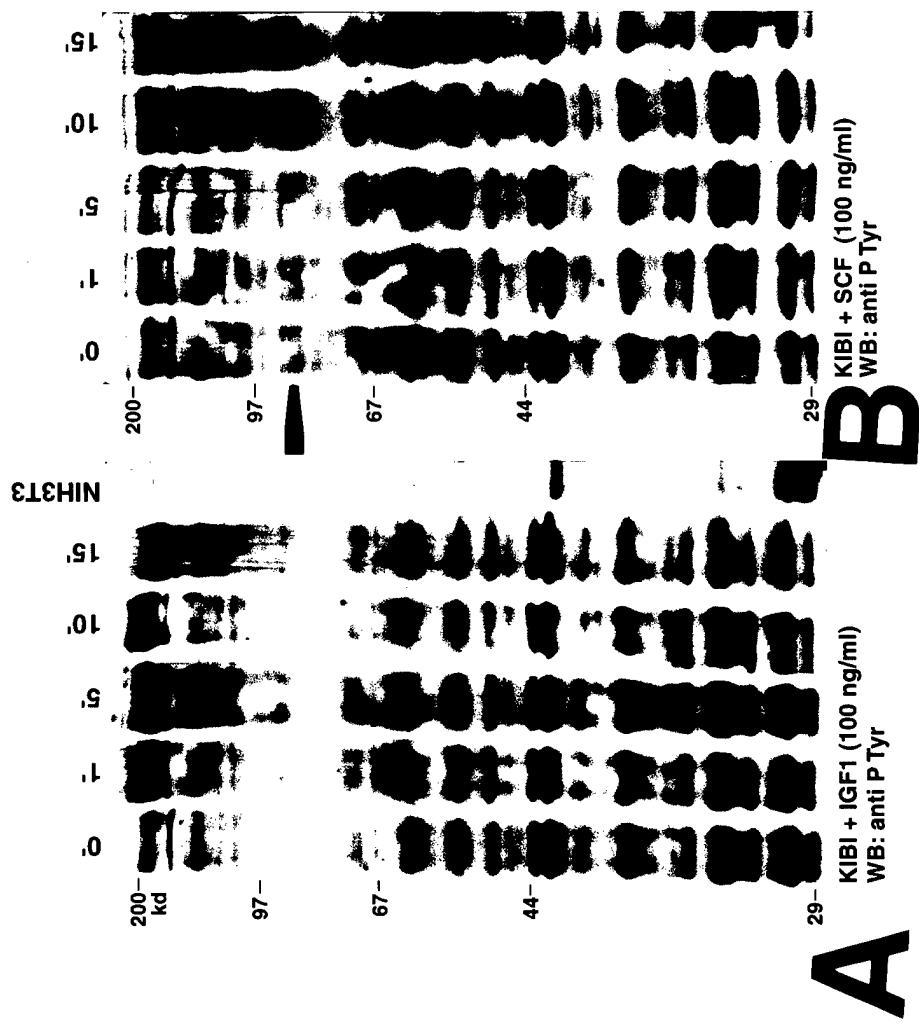
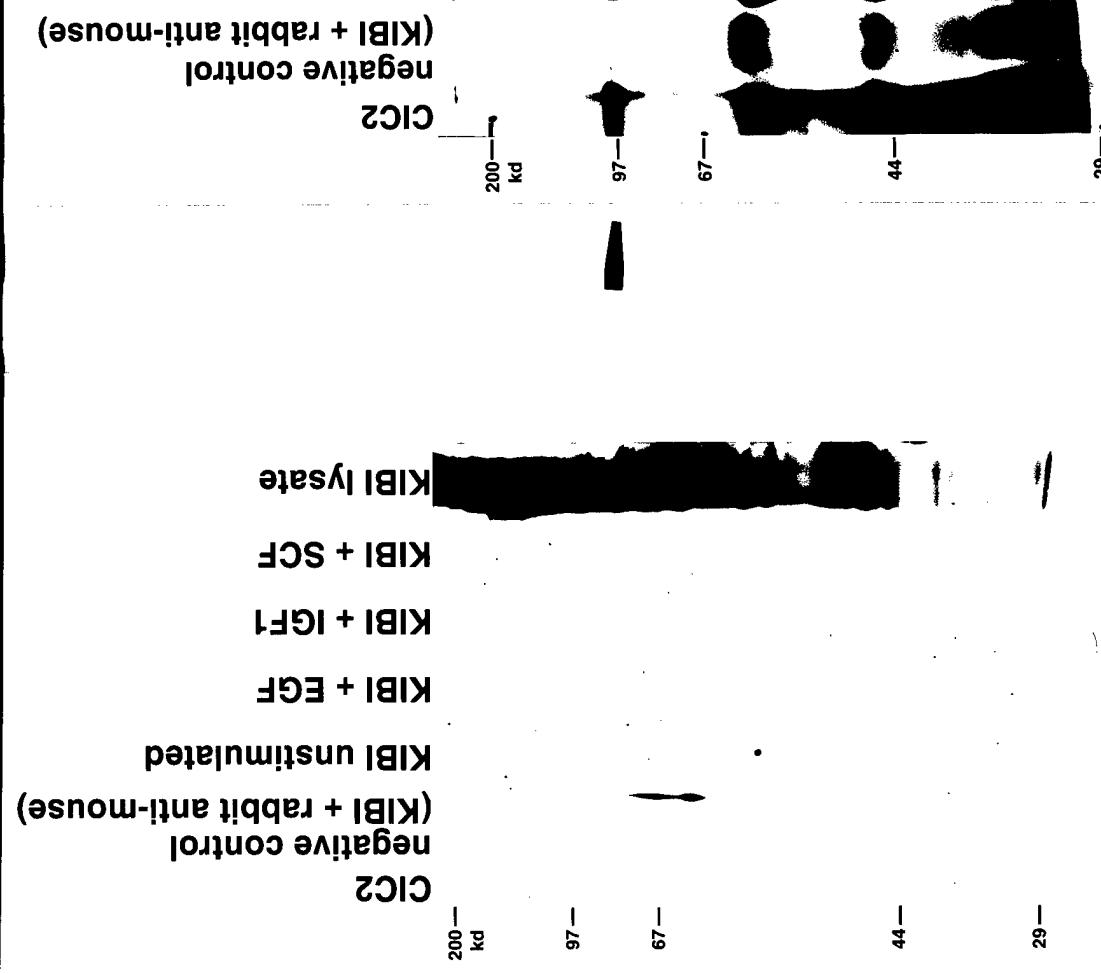


Figure 5. Tyrosine phosphorylation of the KIBI cell line stimulated with either IGF1 or SCF for 0, 5, 10 or 15 minutes. 50 µg of cell lysate was stained with the antiphosphotyrosine antibody -RC20H. **A.** NIH3t3 cell lysate known to express the IGF1 receptor, is used as a control for the IGF1 stimulation. Multiple phosphorylated bands are present, but little phosphorylation is seen in the 90kd area. **B.** In contrast to KIBI cells stimulated with IGF1, SCF stimulation at 10 and 15 minutes reveals a phosphorylated band at a MW of approximately 90kD. This does not appear to be Stat5, but further characterization is underway.

**A IP: anti Stat 5b
WB: anti RC20H**



**B IP: anti Stat 5b
WB: anti Stat 5b**

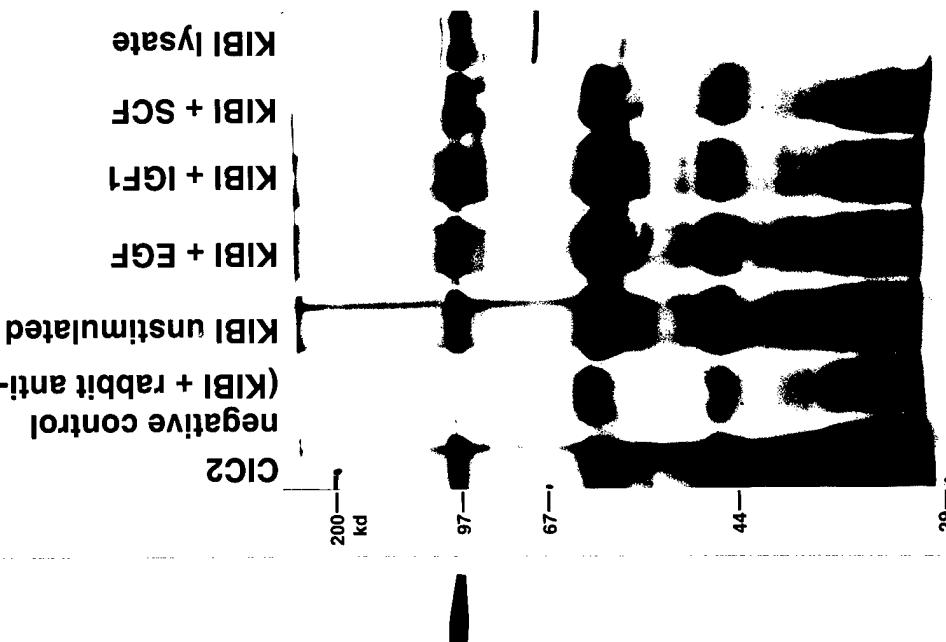


Figure 6. Immunoprecipitation of Stat protein. KIBI cells were stimulated for 15 minutes with either EGF, IGF1 or SCF. 1 mg of cell lysate was immunoprecipitated with a polyclonal antibody directed against Stat 5b. A. Western blot of Stat protein immunoprecipitate stained with an antiphosphotyrosine antibody. No evidence of phosphorylation is seen at a MW of 95 kD. B. The same Western blot as in Figure 6A, stripped and restained with the Stat5b antibody, showing that recovery of Stat protein was successful and quantitative.